Eighth Semiannual Progress Report on

ENZYME ACTIVITY IN TERRESTRIAL SOIL

IN RELATION TO EXPLORATION

OF THE MARTIAN SURFACE

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A.D. McLaren (Principal Investigator)

CONTENTS

I. PREFACE

Our objective is twofold. First, we are developing procedures for detection and assay of enzymes in soil suitable for presumptive tests of life in planetary soils. Incidentally, this requires use of enzyme-substrates that are stable to heat, moisture and storage. We have shown previously that urea, as a substrate for urease, meets these requirements very satisfactory. Second, we are exploring the behavior of enzymes in non-classical (soluble enzyme and insoluble substrate) systems. These include enzyme action at surfaces in gels and in other heterogeneous, structurally restricted systems analogous to those found in cells and in soils.

The Martian environment has a limited moisture content and any biological reactions possibly take place at interfaces and on surfaces in an environment of restricted water availability. A study of surface effects in the hydrolysis of incolubel substrate by adsorbed enzyme (hydrolysis of chitin by chitinase) is being continued in order to investigate some of the factors influencing reactions at inerfaces.

Emphasis has been placed on the detection of urease activity because of the probably primordial origin of urea as an organic substance, because of its stability as an enzyme substrate, and because of the ubiquity of soil urease in the terrestrial environment.

Urease in soils has shown a high resistance to high energy electron-beam irradiation, urease activity has been recovered in over 9000 years old permafrost soil samples, and the hydrolysis of urea by urease in media of low water availability is detectable at 60%

relative humidity and measurable hydrolysis of urea occurs in soils at 80% relative humidity and above. These observations, among others, indicate that there exists in soil an extracellular and enzymologically active moity of urease. Methods are being developed further for their possible use for the detection of such a catalyst in the Martian environment.

Theoretical aspects of the kinetics of enzyme reactions in heterogeneous systems are reviewed and discussed.

PERSONNEL

The participants in the currently reported phase of this project included Professor A.D. McLaren, Dr. J.J. Skujins, Mr. A.H. Pukite, Miss C. Wolf, Mr. W.H. Brams, and Miss Renee Zin-May Sung.

A. SURVEY OF ENZYME ACTION IN HETEROGENEOUS SYSTEMS

Part II

Part I of "Survey of Enzyme Action in Heterogeneous Systems" included sections I, II, and III and appeared in our previous, the Seventh Semiannual Progress Report.

Part II, appearing in this progress report, includes sections IV and V and a bibliography.

OUTLINE OF

"Survey of Enzyme Action in Heterogeneous Systems"

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IV. ENZYME ACTION AT LOW HUMIDITY IN THE SOLID STATE AND IN ICE.

In studies on growth of microorganisms at low humidity in pure culture, on foods, in soil, and for approaches to detection of life on the planets we need to consider questions such as at how low a humidity is cell devision possible, how much water is required for enzyme function, is enzyme action in the frozen sol state observable, as in frozen foods and in permafrost soils etc. (McLaren and Peterson, 1967; Pittendrigh et al., 1966; Meryman, 1966). In all these cases the activity of water may be critical.

A. Studies at low humidity.

In working with dry bacteria, following the addition of water, one must recognize the need to swell the cell so as to expose enzymes held on cell membranes to the action of water and substrates and the possible necessity of diluting any inhibitory substances present in cells. The activation of bacterial enzymes by water can be ascribed to the formation of an active enzyme hydrate. The hydrogenases of some bacteria become active at a water uptake of a few tenths of a mg water per me of dry bacteria, which is about the uptake of water by proteins exposed to one hundred per-cent relative humidity. The catalytic activity of these same bacteria only reaches a maximum "at a concentration of about 3 mg water per mg dry weight of bacteria. It is clear, therefore, that the initial hydration of an active group on the enzyme of the bacteria cannot in itself lead to maximum activity, the latter occurring only when the cell is substantially reconstituted with water ..." (Couper, Eley and Hayward, 1955). The enzyme examined was hydrogenase.

An intimately mixed dry urea-urease powder exposed to air containing descrete amounts of water vapor showed a release of carbon dioxide above a relative humidity of 60 percent. An increase in activity of urease followed the water vapor sorption isotherm of urease and not that of urea. It was found that the minimum amount of water required for activity was 1.3 moles per mole of side chain polar groups of the urease protein (Skujins and McLaren, 1967). Soils exhibit urease activity and these results show that considerable hydrolysis of urea could occur in "air-dry" soils at 0.8 relative vapor pressure and above, with a maximum at unit activity of water.

Meats must be stored below 70 percent relative humidity in order to avoid enzymatic spoilage. In fact amylase activity in dried foodstuffs can take place even as low as at 36 percent R.H. provided a fine capillary structure is present in which moisture may condense (Sharp and Rolfe, 1958).

It would be most worthwhile to know these humidity limits for enzymes not involving water as one of the stoichemetric reactants and to study the migration of water molecules from polar sites to substrates for all enzymes in the absence of liquid water. Such studies could contribute to the mechanism of enzyme action in general.

B. Studies with solid enzymes.

The observation that substrates can, in certain instances, diffuse into crystals of enzymes, can now be extended to explore the structure of enzymes and the mechanism of enzyme action (Doscher and Richards, 1963; Wychott et al., 1967). In the case of ribonuclease-S, the crystal lattice

seems to act as a molecular and information can be deduced as to the number of enzyme binding sites and to ascertain whether different ligands compete for the same sites by X-ray analysis. It appears that catalytic activity is a property of enzyme molecules throughout the crystal and without disruption of the lattice. Structural aspects of ribonuclease-S related to the active site are not markedly changed as enzyme molecules are dissolved. These observations apply to low molecular weight substrates such as cytidine 2', 3'-phosphate. Yeast RNA hydrolysis probably involves enzyme molecules at the surfaces of crystals.

The substrate carbobenzyoxyglycyl-L-phenylalanine has been passed through a column containing crystals of carboxypeptidase-A, crosslinked with glutaraldehyde to give an insoluble catalyst. The preparation is much less active than the soluble enzyme and the activity of a similar, amorphous enzyme preparation was intermediate. An absence of diffusion-limiatation of reaction kinetics were noted provided the crystals were small. (The concentration of half maximum activity for all samples of forms of carboxypeptidase-A were in the range 0.01-0.02 M.)

For the simple model of diffusion into a thick slab coupled with chemical reaction, Doscher and Richards predict that the steady-state rate will be a linear function of the substrate concentration in the external liquid provided this value is well below the Michaelis constant for the crystalline enzyme. At concentrations above K_m the rate increases with a fractional power of substrate concentration, rather than being independent of it as with solution kinetics.

In a related study, Kallos ( ) has measured the activity of crystals of chymotrypsin toward acetyl-L-tyrosine hydrazide and acetyl-L-tyrosine ethyl ester as substrates in 80 percent saturated ammonium sulfate. The solid enzyme had about 20 percent of the activity of soluble enzyme in both cases, suggesting that the crystalline enzyme was in an active form.

C. Reactions in ice and frozen foods.

Tappel has summarized current knowledge of the kinetics of enzymes in frozen systems ( ). Slow freezing gives rise to localized concentrations of substrate and enzyme in small liquid pools; this localization gives rise to the more rapid rate of the frozen system over supercooled solutions at -4.2°C. When rapidly frozen the reactions remain homogeneous and initial rates of the peroxidase-catalysed oxidation of quiacol are less than that in supercooled liquid. Unlike the reaction in ice, reactions in supercooled liquids proceed until the reactants are exhausted. Many factors can contribute to the increase in Arrheimus factors below freezing temperatures such as polymerization of enzyme, changes in the ionization of all species, conversion of enzymes to alternate conformational isomers, etc. Qualitative studies on many enzymes are summarized in a new monograph (Meryman).

Freezing has been observed to accelerate other catalysed reactions, including hydrolysis, aminolysis, oxidation and peroxide decomposition. With benzoyl-L-argenine ethyl ester, the rate of formation of the corresponding hydroxamic acid catalysed by trypsin at 1°C initially

exceeded that at -18° but then became less as the nature of the reaction changed to hydrolysis to the amino acid. Thus an enzyme reaction may both change velocity and chemical pathway following freezing of the system (Grant and Alburn, 1966). For a summary of pioneer work in this area see Joslyn (1949).

Invertase action has been observed at -18° in fruits but not at -40°C (Joslyn and Marsh, 1933). It has been suggested that ice per se has catalytic properties, however (Grant, 1966), as for example in promoting the hydrolysis of glutamine, but suitable controls may not always be conceptually clear. Tissues present formidably complex systems for analysis.

V. COMPARISON OF SOME NATURALLY OCCURRING BOUND ENZYMES WITH SOLUBILIZED ENZYMES.

Considerable interest in the localization of enzymes in and on cells (Holter, 1952) (Alexander, 1956) (Rothstein, 1954) (Krebs, 1962) is being shown. In this section examples will be cited to illustrate applicability of some of the theoretical features encountered above.

A. Cell surface enzymes.

Attention has been called to the similarity between the pH activity curves of certain enzymes reactions in intact cells and mitochondria and those observed in solution (Rothstein, 1954). Because of this similarity it has been suggested that with trehalase, lactase, and invertase of yeast that the enzymes concerned must be peripherally located in the cell.

The assumptions are (i) that the internal pH of the cell is almost independent of the external pH of ambient buffer, (ii) that the permeability of the cell membrane to substrate is independent of pH, and (iii) that the extremes of pH do not kill cells. These assumptions are all valid with yeast (Rothstein, 1954, Wilkes and Palmer, 1932). Since a charged surface of a cell will either attract or repel hydrogen ions, depending on the sign of the charge, an enzyme acting on the surface will be exposed to, and perhaps in equilibrium with a hydrogen-ion activity differing from that of ambient solution. In Fig. 2 are plotted data from the paper of Wilkes and Palmer (1932) for the effect of pH on invertase activity of yeast cells and of isolated enzyme, with the expected ΔpH. More recent work with a different approach has verified the conclusion that invertase is located on the surface of yeast cells (Dennis et al., 1954).

Burstrom has observed a splitting of sucrose by wheat roots; the hydrolysis proceeds by enzyme action at the root surface. The root surface is negatively charged and one way of decreasing ΔpH is by increasing the external salt concentration at constant pH 6. A comparison of columns 2 and 3 in Table VII shows "that the rate of hydrolysis closely follows the hydrogen ion concentration on the root surface, independently of the external pH." Root enzymes, such as surface (epidermal) phosphatase exhibit typical Michaelis-Menten kinetics, equation 6, in vivo as tested with excised tissue (Estermann and McLaren, 1961).

B. Subcellular enzymes

1. Some remarks on cell pH and soluble enzymes.

Some cellular enzymes are in the fluid portion of cells (Holter). These are exposed to the intracellular pH and redox potential and in large cells this may have some average meaning. The intracellular pH (pH_i) of (resting) S. cerevisiae is 5.8 as a whole and the buffering power of the cell is considerable. Thus the enzyme system fermenting glucose is nearly independent of pH_b over a wide range (Ingram, 1955). The buffering power resides more in salts than proteins, and on prolonged fermentation the interior pH value may exceed 6 whereas at the outer layers a drop to 4.2 may occur.

Considering the rate of reaction in the steady state of reduced pyridine nucleotides in yeast, and assuming that K for the reaction

$$K = \frac{(H^+)(DPNH)(\text{acetaldehyde})}{(DPN^+)(\text{ethanol})}$$

is the same in vivo as in vitro, chance calculated an intracellular pH about two units higher than expected from solution studies ( ).

The action of xanthine oxidase has been compared in a normal environment, lymphocytes, with action in a synthetic medium following cellular destruction. Since the Arrhenius activation energy was about the same with cells (20,000 cal/mole) and in solution (13,300 cal/mole) and in the range of that normally found with enzymes, it was concluded that passive transport of the substrate, xanthine across the cell membranes was not rate-limiting. For intact cells K_m was 1.8×10^{-5} and in solution K_m was 2.0×10^{-5}; Michaelis-Menten

kinetics were observed in both experiments. These results (Ultmann et al., 1960) can be interpreted to mean that some cellular enzymes are in micro-environments not differing drastically from test-tube experiments.

On the other hand, many enzymes in cells are associated for the most part with specialized structures, the inner cell membranes, reticular networks (giving rise to microsomes by rupture), mitochondria, etc. (Hodge et al., 1957). If this were not the case it is difficult to see how reaction sequence could be controlled (Schneider et al., 1955). The state of the enzyme in or on such a particle seems to determine, for example, the pH optima of conjugated (pH = 7-8) and dissociated (pH = 9.5) malic oxidase still attached to particles of the cyclophorase system (Huennekens, 1951).

The meaning of pH in small particles breaks down as pH is a statistical concept (Baum, 1967). For example, a particle 0.5 μ in diameter with a continuum of pH throughout the interior and its surroundings of $pH_b = 7$ would have room for only about 4 hydrogen ions. If the particle contained protein, then the probability that an active center of an enzyme involving a carboxyl group would be ionized may depend on how close to the -COOH group may be located one or more ammonium groups. These charged ammonium groups could repel hydrogen ions and alter the pK of the carboxyl group and hence the pH optimum exhibited by the enzyme. Any reorganization of the protein make-up of the particle, such as by freezing or thawing could change the pH optimum by changing the relative positions of

the ionizing groups which are parts of the active centers involved in enzyme action. Such a consideration may account for the difference in pH optimum of aconitase in solution and as part of intact mitochondria of Lupinus albus (Estermann, et al.). The difference is just opposite to that expected from the electrophoretic data of Table I, assuming the enzyme to be only on the surface of mitochondria. That is, the pH optimum of the aconitase in the mitochondria is below pH = 6 whereas that of the solubilized enzyme is above pH = 7, although the particle is negatively charged externally. Ergo, the enzyme is not acting at the surface of the particle.

The fact that aconitase in lupine mitochondria has a pH optimum of 5.8 while the enzyme extracted out has one at 7.0 (Estermann et al.) could be also due to the pH activity curve of the enzyme per se being superimposed on the pH curve of the permeability curve of substrate into mitochondria, however (Siekevitz, 1962).

Incidentally, it is frequently the practice to fractionate cellular homogenates and to attempt to account quantitatively for the distribution of an enzyme among the fractions by observations of enzyme activity at a given pH_b. If the pH optimum is different from one surface or particulate to another, obviously the total activity of the intact tissue will not equal the sum of the individual activities as measured at a fixed pH_b.

In bacteria the difficulty of defining a pH_i is even more acute, although operational values can be assigned, based on indirect arguments (Dewey, 1966, Pardee et al.). Certainly the growths of

bacteria are responsive to ambient pH, even though the bacterial cell has a way of maintaining a pH_i more or less independent of external pH (Dewey).

At the present time the problems of the meaning and determination of intracellular pH even of cells of moderate size are still being explored. That a point to point variation of hydrion "concentration" exists is most probable. Katchalsky and colleagues have been studying the potentiometric behavior of simple gels which can be characterized by two factors -- the electrostatic interaction among the ionic constituents and the contractility of the polymer network. These studies should provide useful models for describing cell structures of interest to the enzymologist. In substance, in order to relate the pH of bulk solution to the degree of ionization, α, of the gel network and the number of small ions in the gel per monomer unit, p, Katchalsky gives

$$pH_b - pH_g = 1/2 \log \frac{[X_g^+]}{[X_g^-]} = 1/2 \log \frac{p + \alpha}{p - \alpha} \qquad (4)$$

The subscript, g, applies to the gel and $X^{\pm}$ represents molal fractions of univalent small ions. pH differences between the two phases can thus be evaluated from ionic concentrations. The differences were found to be of the order of 0.2 to 1.2 depending on the ionic strength of the external solution. Although Donnan's theory cannot be expected to apply well to living cells, since they are not at thermodynamic equilibrium, such equations may be pertinent to two phase systems within a cell (Michael and Katchalski, 1957).

In a review, Shugar has called attention to the possible influence of Δ pH on the proper choice of conditions for estimating enzyme action in thin tissue sections with histochemical techniques (Shugar).

2. Kinetics with particulate enzymes.

Ernster and Lindberg ( ) offered two explanations for their observation that hexokinase has a higher activity in the presence of (absorbed on ?) mitochondria. If the hexokinase exhibits its activity on the mitochondrial surface, the local concentration of ATP may be increased by a spacial arrangement. Or, "working mitochondria activate hexokinase". Siekevitz et al. (1959) also observed that yeast hexokinase bound to liver mitochondria can be many times more reactive than the free form. An attempt to duplicate the phenomenon by substituting paraffin-spheres bearing surface charges in place of mitochondria failed: all of these "model mitochondria", when added to a hexokinase - substrate system, depressed the enzyme activity. Perhaps the mitochondria are capable of a specific rather than simple electrostatic interaction with the enzyme (Ruchti and McLaren, 1965).

A number of cases are known in which an enzyme activity occurs in both the soluble and mitochondrial fraction of a homogenate and in which physical or kinetic properties support the notion that these are identical species: aconitase in lupine mitochondria (Estermann, et al.), liver alkaline ribonuclease (Beard and Razzell, 1964), liver isocitric dehydrogenase (Henderson, 1965), liver diapharase (Conover and Ernst, 1962), and mitochondrial hexokinase (Rose and Warms, 1967). A reversible release of hexokinase from ascites tumor mitochondria

suggests that a natural equilibrium pertains (Rose and Warms, 1967). Chymotrypsin causes a loss in binding capacity of the enzyme to the particulate without loss in the catalytic property which indicates a specific binding role for part of the hexokinase molecule. Added glycerol kinase competes successfully with bound hexokinase for ATP, whether it is derived from oxidative phosphorylation or from reaction with added creatine kinase; this suggests that hexokinase acts on the ATP after it has left the intramitochondrial region of oxidative phosphorylation. Here vector chemistry is manifestly involved. Hexokinase kinetics depend on the state of the mitochondrial envelope (Li and Chien, 1966) and on the binding (Table V).

A detailed comparison of the kinetic behavior of an enzyme in an isolated, soluble state with its behavior in an insoluble, organized particulate is that of Gawron et al.

The reaction system chosen is the following.

$$\underset{(S)}{\text{L - Chlorosuccinate}} + \underset{(A)}{\text{ferricynide}} \longrightarrow \text{Chlorofumarate}$$

Initial velocities, v, were measured with soluble and with particulate enzyme.

1/v vs. 1/S plots were linear in both cases but differ in effect of ferricyanide on the slopes of the plots. The initial rate data and kinetic relationships are accounted by assuming that both reactions 3 and 4 below must be taken into account with soluble enzyme while the last reaction is predominant in the particulate system, viz,

$$E + S \rightleftharpoons ES \quad (1)$$

$$ES \rightleftharpoons E''P \quad (2)$$

$$E''P + A \rightleftharpoons EP + A_{red} \quad (3)$$

$$E'' + A \rightleftharpoons E + A_{red} \quad (4)$$

where E'' and E''P are reduced enzyme species.

In one reaction the reduced enzyme is oxidized by ferricyanide after releasing the product, in the other reaction, before. Evidentally the diffusion of oxidant to the enzymatic site is rate limiting with the particulate enzyme. [Cf. Minkami et al.J.B.C. 237, 569, part in bibliography, (1962)].

The addition of calcium ions to mitochondria suspensions containing succinate can cause a more rapid oxygen uptake and a permeability change. Thus, although fumarate is not freely available to the dehydrogenase in intact mitochondria, it becomes freely available as a competitive inhibitor of the dehydrogenase in calcium chloride-treated mitochondria (Gutfreund and Jones, 1964). Permeability also plays a major role in differences of fumarase activity in intact and permeable mitochondria, as do differences in internal ionic strength and phosphate concentration (Alberty et al., 1954).

It has been found that treatment of mitochondria with C_{12} fatty acid (dodecamate) removes the internal structure and at the same time eliminates the oxidation of pyruvate by the enzymes of the citric acid cycle. This additional fact stresses the importance of structure in some organized actions of enzyme systems (Baker et al., 1962. The subject has been reviewed by Lehninger, 1966).

The maximum allowable cell size or volume element length between two enzymes acting on a common intermediate has been calculated from elementary principles by Weisz (1962). The length depends on the concentration and on enzyme turn-over numbers and it falls in the range 10^{-3} to 10^{-5} cm , that is lengths smaller than most bacterial cell dimensions and up to and including many plant and animal cells. Many factors can modify these estimates, such as leakage from the system, variation of diffusion coefficients from point to point, branching of metabolic pathways, feedback inhibition, etc. (Pardee, 1962) (Blum and Jenden, 1956).

As substrates increase in molecular size, the sieve action of polymeric networks in cells can be expected to reduce the collision frequencies with enzymes and co-factors, and as we have seen with the trypsin-gelatin system, one manifestation can be an increase in K_m. An extreme case of this kind has been analysed by Ts'o and Lubell (1960). They looked into the theory that S-RNA carries the activated amino acids to the microsomal particles (p) in hemoglobin synthesis. The concentration of particles and S-RNA in the cell was found to be 3×10^{-6} and 6×10^{-5} M, respectively. Collision frequency, z, was calculated from Smoluchowski's equation. It is given as

$$z = 2 \pi (D_p + D_r) N_p N_r (R_p + R_r)$$

where D_p and D_r are the corresponding diffusion coefficients inside the reticulocytes, N_p and N_r are the numbers of molecules per cubic centimeter, and R_p and R_s are the effective collision radii. (A term involving the square root of the diffusion coefficient was

discarded as insignificant.) For S-RNA, the radius of gyration of a random coil was used. In calculating diffusion coefficients a relative viscosity of 2.1 was utilized, based on viscosity measurements of the cell fluid. Assuming that the components exist in free form inside the cell and that they do not have any specific interactions, which seems to be the case, z was calculated to be 2.2×10^{20} sec./cc collisions between the particles and S-RNA.

The number of peptide bonds formed in the particles per second per cc of cell volume is only 1.1×10^{15}. (There are 1.4×10^{15} particles per cc and each peptide bond requires 1.3 sec. to be formed in the particles.) Now, if only 10 percent of the microsomal particles are active, and if twenty amino acids are involved, and in the proper order, the collision efficiency is reduced to only 0.5 percent of that calculated. There is also the requirement that enzymes, Mg ions, nucleotide triphosphates etc. be present in non-rate limiting amounts. But if more than 0.01 percent of the collisions result in amino acid transfer, the collision frequency is sufficient to support the rate of peptide-bond synthesis which has been observed. This story has been extended by Pollard (1963) to show that in cases where collision numbers are too small, cells with dimensions in excess of one micron will require some mechanism to guarantee collision: "The presence of an activation energy will require that successive reactions be produced in an organized system."

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II EXPERIMENTAL

A. STUDY OF ORGANIC MATTER EXHAUSTED SOILS.

This report describes the continuation of work initiated on organic-matter exhausted soils (1).

Jenkinson (2) followed the mineralization of organic matter in soils after repeated treatments consisting of a partial sterilization by heating or by chloroform vapor followed by inoculation with soil organisms. With successive treatments the amount of organic matter mineralized gradually decreased but even after five treatments less than five per cent of the total organic matter had been mineralized, indicating that a large portion of the organic matter consisted of material that was not degradable by microbial action nor was rendered susceptible to microbial degradation by the treatments used.

The object of the study is to determine whether there exists in soils an organic matter fraction having enzymatic activity that is resistant to the degradative activities of the soil microorganisms. In this study it is important to differentiate between enzymatic activity residing in this resistant fraction and the activity arising from the microorganisms in the soil or from material susceptible to microbial degradation. Therefore, it is necessary to remove soil organisms and their associated enzymes. The method used must not destroy the enzymes that are resistant to degradation. The method used to meet these objectives is alternately to sterilize the soil and then to reinoculate and incubate it. Sterilization will kill the soil microorganisms and

inoculation and incubation will allow the organisms in the inoculum to feed upon the killed cells and susceptible enzymes. Repetitions of these treatments will yield a soil in which the original microbial population and susceptible enzymes have been removed, leaving the organic fraction that is resistant to the degradative activities of the soil microorganisms. The hydrolysis of urea to ammonia and carbon dioxide by urease was chosen as the enzymatic activity to be followed.

The goals of the present study include determination of the number of viable microorganisms remaining in the soils after they have received chloroform treatments, to determine the efficacy of these treatments alone in sterilizing the soils, and to decide whether the present plating medium is sufficient to support the growth of microorganisms in the treated soils.

Materials and Methods

Soils: The soils were obtained from Dr. D.S. Jenkinson, Rothamsted Experiment Station, England, and they were the ones used in his experiments (2); they had the code letter "c". We used a sample of untreated "c" soil and a sample of "c" soil which had undergone five treatments of chloroform sterilization, inoculation, remoistening and incubation, the fifth treatment followed by a final inoculation, incubation for ten days and drying. These two Rothamsted soils designated "untreated" and "treated" in the previous report (1) are designated "X" and "CV", respectively, in the present work.

Soil samples contained in beakers were crushed to a fine powder under sterile conditions using a glass stirring rod.

Dublin soil was a dry sieved soil that had been stored air-dry for 6 years. Oxford soil was obtained from the Oxford Tract, University of California, Berkeley, air-dired crushed and sieved (1).

Chloroform treatment: A treatment similar to that of Jenkinson is used (2). The soils are placed in small beakers, moistened with sterile water to a water content of about 30%, and placed in a dessicater lined with wet filter paper and containing a beaker of chloroform treated to remove its ethanol additive (3).

The dessicator is evacuated by a water aspirator until the chloroform begins to boil and then it is sealed. After a few minutes the dessicator is again evacuated without allowing air to enter until the chloroform begins to boil, and this process of successive evacuations is repeated several times until all the air inside the dessicator has been replaced by water and chloroform vapor. The dessicator is placed in the dark at room temperature. Twenty-four hours later the dessicator is opened, the beaker of chloroform removed and the treated samples are dried in a stream of filtered air to prevent contamination by air-borne microorganisms.

Samples receiving repeated chloroform treatments without incubations were treated as usual, and the air-dried treated soil was used as the soil sample for the next treatment.

Inoculation and incubation: Samples of treated X soils are inoculated by untreated X soil, and samples of treated CV soil are inoculated by untreated CV soil. Fifty mg of untreated soil is shaken with 5.0 ml of sterile water, let settle one minute, and the suspension is used to inoculate the soil sample. The soil samples are inoculated to a water content of 30% and stirred to distribute the inoculum uniformly. Each inoculated soil sample is placed in a 500 ml capacity wide-mouth Mason jar which contained 5 ml of water and a test tube containing 5 ml 2N NaOH solution. The caps are screwed on tightly and the jars are placed in the dark at room temperature. The soils are incubated for ten days then are removed and dried under a stream of filtered air.

Plating media: A basic mineral salts medium consisting of 0.05% KH_2PO_4 and 0.02% $MgSO_4$ was prepared to which nutrients were added to give a complete growth medium. Difco peptone and Difco yeast extract, each at 0.005% or at 0.01%, gave 0.005% PYE and 0.01% PYE media respectively. The former medium, in which 0.005% Difco Casamino acids replaced the peptone, gave 0.005% AA-YE medium. Soil extract was prepared from Dublin soil according to the method of Pramer and Schmidt (4) and $MgSO_4$, KH_2PO_4, and NH_4Cl were added to the extract to 0.02%, 0.05% and 0.01% respectively, giving a fortified soil extract. This was diluted with the basic mineral salts medium to which NH_4Cl had been added to 0.01% to give 0.1%, 1.0%, 10%, and 50% concentrations of fortified soil extract in the augmented mineral base (SE-base media). Similarly, fortified soil extract was diluted with 0.01% PYE medium to give SE-PYE media. The fortified soil extract alone was also used as a

plating medium. All media were brought to pH 7.0 with NaOH and Difco agar was added to 1.5% prior to sterilization to prepare the complete plating medium.

Urease activity was determined by the analysis of released $C^{14}O_2$ from C^{14}-labelled urea as described in previous reports (1). To a 1 g soil sample in a planchet was added 10 mg C^{14}-urea (Calbiochem, Los Angeles) containing 10 μc C^{14}, and 0.5 ml K-acetate, pH 5.5, 0.05 M. The planchet was placed in a radioactive gas counting chamber and the increase of $C^{14}O_2$ in the chamber was monitored with a Geiger-Mueller gas-flow tube connected to a decade scaler and to a count ratemeter. Integrated amounts of $C^{14}O_2$ in the chamber were strip-chart recorded. Results were expressed as the rate of increase of counts per minute (Δ cpm/m) during the first 100 minutes.

Results

The results of various chloroform treatments on the microbial numbers and urease activity in the tested soils are given in Tables I to VI.

Table I

Microbial numbers in soils receiving alternating chloroform treatments and incubations.

Treatment	colonies/g		urease activity Δ cpm/m/g	
	X	CV	X	CV
soils as received	2.82×10^6	3.04×10^7	3.35	.461
after one $CHCl_3$ treatment	5.62×10^5	2.96×10^7	2.52	.360
above, plus incubation	2.69×10^7	5.66×10^7	0.89	.610
above, plus 2nd $CHCl_3$ treatment	3.30×10^7	8.84×10^7	–	–

Table II

Microbial numbers in uncrushed and crushed soils.

Treatment	colonies/g	
	X	CV
soils as received	4.62×10^6	4.97×10^7
crushed soil	6.40×10^6	6.11×10^7

Data presented in Tables I and II were obtained by strictly following Jenkinson's procedure (2), i.e., the dessicators were not completely evacuated and chloroform was not brought to boiling. Data presented in Table III *et seq.* was obtained by using chloroform treatment as described in "Methods" above.

Table III

Microbial numbers in crushed soils receiving chloroform treatment as described.

Treatment	colonies/g	
	X	CV
soils as received	6.40×10^6	6.11×10^7
after one $CHCl_3$ treatment	1.05×10^6	5.72×10^7

Table IV

Microbial numbers in crushed soils receiving repeated chloroform treatments.

Treatment	colonies/g	
	X	CV
soils as received, crushed	6.40×10^6	6.11×10^7
after one $CHCl_3$ treatment	1.05×10^6	5.72×10^7
above, plus incubation	7.97×10^7	8.23×10^7
above, plus 2nd $CHCl_3$ treatment	3.72×10^7	6.13×10^7
above, plus incubation	4.04×10^7	5.00×10^7

Table V

Microbial numbers in soils receiving repeated chloroform treatments without intervening incubations.

Treatments	colonies/g	
	X	CV
soils as received, crushed	6.40×10^6	6.11×10^7
after one $CHCl_3$ treatment	1.20×10^6	8.25×10^7
after two $CHCl_3$ treatments	6.02×10^5	5.20×10^7
after three $CHCl_3$ treatments	3.94×10^5	3.00×10^7

Table VI

Effect of plating medium on numbers of soil organisms counted.

Medium	Organisms/g x 10^{-6}	
	Dublin	Oxford
0.005% AA-YE	4.31	10.9
0.005% PYE	4.72	8.8
0.01% PYE	4.57	9.3
0.1% SE-PYE	4.77	11.6
0.1% SE-base	4.07*	4.5*
1.0% SE-PYE	4.22	8.9
1.0% SE-base	4.07*	7.3*
10% SE-PYE	4.94	9.4
10% SE-base	4.81*	8.5
50% SE-PYE	4.94	13.4
50% SE-base	5.20	12.6
100% SE	4.83	10.0
Average	4.62	9.6

* colonies were smaller than on a richer media.

Discussion

The chloroform treatments partially sterilized the soils and caused a decrease in the urease activity of the soil (Tables I and III). The decrease in the number of countable organisms differed for the two soils. For uncrushed soil X, 20% of the organisms remained countable whereas for uncrushed soil CV 97.4% of the organisms remained countable. Using crushed samples the values were 16.4% and 93.7% respectively. Since CV soil had received five chloroform treatments by Jenkinson prior to its use by us, its microbial population may consist mostly of chloroform-resistant organisms, the sensitive ones having been killed by his treatments. Therefore, an additional chloroform treatment by us would kill relatively few organisms. This explanation is confirmed by the data above and also by the values for microbial numbers following the second chloroform treatment. After the second treatment the numbers did not decrease, but in fact increased (23% and 56% for X and CV soils respectively). The cause of the increase is obscure but may be due to the presence of air during the chloroform treatment.

Incubation of the chloroform-treated soils caused an increase in the microbial population of the soils. For X soil the increase was 9.54-fold; for CV soil the increase was 1.86-fold, as calculated on the basis of the numbers of organisms in the soils as received. The reason for the smaller increase in the case of CV soil sample may be that the treatments given to this soil by Jenkinson had resulted in the exhaustion of readily available nutrient material so that only a limited

increase in microbial numbers could occur upon incubation. Also, his treatments may have selected for a microbial population that can utilize the soil organic matter for growth and multiplication only poorly.

After each treatment the urease activity of the soils decreased (except for CV soil after incubation) indicating possible inactivation of urease by chloroform treatment.

The number of microorganisms in crushed soils was higher than that in uncrushed soils (Table II). For X soil it was 40% higher and for CV soil it was 22% higher. Uncrushed soil contains crumbs of soil which may not be completely disintegrated during the suspension of the soil for plating. Since the release of the microorganisms into the water used in the soil dilutions is hindered, underestimation of the number of soil organisms will result. In taking soil samples for plate counts care was always taken to include both the fine material and the crumbs in constant proportion in order to minimize errors due to variation in crumb size.

The characteristics of the microbial population in the crumbs is similar to that in the fine material of the soil since the decrease in countable organisms after chloroform treatment in the crushed soil is similar to that of the uncrushed soil; namely, 16.4% vs. 20% for X soil, and 93.7% vs. 97.4% for CV soil (Table III). The decrease in the number of countable organisms are slightly greater for the crushed soil because the modified procedure (namely, as described in "Materials and Methods" above) of chloroform treatment was used for this soil.

It should be noted that according to Jenkinson's method (2) complete evacuation of the incubation chamber was not achieved, and, apparently, the atmosphere was not chloroform-vapor saturated. In our modification the chloroform vapor is undiluted by any air remaining in the dessicator and consequently acts more thoroughly as a sterilizing agent.

The repetitions of chloroform sterilization and subsequent reinoculation caused the same changes in microbial numbers as were observed before (Table IV). The first chloroform treatment caused the microbial numbers to decrease. After inoculation and incubation the numbers rose to values exceeding those in the soils initially. Further treatments caused little change in the numbers. The results were similar quantitatively to those observed before, indicating that modification of the chloroform treatment did not change its effect greatly.

After the first incubation, further chloroform treatments caused little change in microbial numbers suggesting that the first chloroform treatment killed all chloroform-sensitive microorganisms and left a chloroform-resistant fraction. This hypothesis is supported by the data in Table V. After the first chloroform treatment further chloroform treatments (without intervining incubations) caused little decrease in microbial numbers. This would indicate that the method of chloroform sterilization is not general as it acts effectively only upon a specific fraction of soil microorganisms. Also, for the microbial fraction resistant to its effects the method of sterilization is relatively ineffective, causing reductions of only 50% in numbers after each treatment.

After the first incubation the microbial numbers did not increase with subsequent incubations. Perhaps after the first flush of multiplication little nutrient material remains in the soil to support multiplication or the microorganisms are unable to utilize the organic matter in soil for growth either because the organic matter that remains is incapable of supporting multiplication or because the surviving organisms themselves do not have the metabolic capacity to utilize the organic matter. The treatments themselves (wetting, drying, inoculation, exposure to chloroform vapor) apparently do not produce nutrients from the soil organic matter capable of supporting cell multiplication. The value of about 8×10^7 organisms/g soil may represent the point at which inhibitions of growth by organisms on one another become effective, placing a limit to the extent of multiplication possible in the soil samples.

Since the source of nutrients for organisms growing in unamended soils is the organic matter of the soil itself, the plating medium that would support the growth of the widest range of microbial types and would best support their growth might be one that included nutrients derived from soil. However, since sterilizing procedures may affect the metabolic capabilities of surviving organisms, leading to a requirement of certain factors that are normally not required by the unaffected cells, the medium must contain a wide range of nutrients.

To explore the effect of plating medium on the number of microorganisms that give rise to countable colonies on agar plates, a

variety of media were used to determine the counts of microorganisms in two different soils. The results are shown in Table VI.

Media with low concentrations of soil extract in mineral base gave low counts and smaller colonies compared to those obtained with the regular plating medium, indicating that these media are poorer than the regular plating medium. The various media containing about the same concentration of nutrients as the regular medium gave about the same counts. Media with high concentrations of soil extract gave higher counts, compared to the control, about as much as 15% higher for Dublin soil and 35% higher for Oxford soil. The effect on the numbers may be due either to a higher concentration or to a greater variety of nutrients, leading to the growth of microbial types that would form only tiny colonies on the regular plating medium. However, these increases may be an artifact of the experiment since halving the concentration of the regular plating medium or replacing the peptone by amino acids did not greatly affect the numbers. The data are insufficient to indicate whether high concentrations of soil extract lead to the growth of microbial types that would not form colonies on the regular plating medium. In any event, the effect is small and it appears that soil extract need not be an essential ingredient of the plating medium.

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B. PERSISTENCE OF UREASE ACTIVITY IN IRRADIATED SOILS.

As continuation of our previous studies on the persistence of urease activity in soils (1) we have examined the effects of large dosages of high-energy radiation on the urease activity in several latosols.

Materials and Methods

Soils. The Dublin soil has been stored air-dry at room temperature for 12 years.

The Hawaiian latosol samples were obtained through the courtesy of Dr. O.R. Younge from the Department of Agronomy and Soil Science, University of Hawaii, in 1964. The Puerto Rico latosol Nipe clay was obtained through the courtesy of Dr. R.A. Luse, Agricultural and Biosciences Division, Puerto Rico Nuclear Center.

The descriptions of soils are summarized in Table VII.

The soil organic carbon content, pH and the enumeration of organisms has been described in the previous report (1).

Urease activity determination in soils was based on the detection of $C^{14}O_2$ released from C^{14}-labelled urea-ammended soils as described in detail before (1).

Table VII

Description of Soils

Soil Sample	Description
Dublin	Contra Costa County, California, adobe clay loam, A horizon, stered 12 years.
Kawaihae	Island of Hawaii, Red Desert latosol loam, top 5 cm.
Lahaina	Island of Oahu, latosol clay, top 25 cm.
Mahukona	Island of Hawaii, latosol silty clay loam, top 15 cm.
Wahiawa	Island of Oahu, latosol clay, top 25 cm.
Molokai	Island of Oahu, latosol clay, top 25 cm.
Nipe I	Puerto Rico, latosol clay, top 2.5 cm.
Nipe II	Same as Nipe I, 15-25 cm deep.

Irradiation with an electron beam were carried out as described previously by McLaren et al. (1962) with the LINAC electron accelerator of Lawrence Radiation Laboratory, University of California, operating at 5 Mev.

The soil to be irradiated was sealed in polyethylene bags made of polyethylene tubing of 0.005 inch thickness. The bags were placed on a plywood disc, rotating in front of the beam (20 rpm), held taut and the soil was pressed against the rim so that its maximum thickness did not exceed 2 cm; this ensured penetration of beam energy through the entire depth of soil. A temperature rise of only 7^{o} to 8^{o}C was noted in the soil during irradiation.

Absorbed doses in the 10^3 to 10^7 rad range were determined by a measurement of the change in optical denisty of cobalt glass by a suitable spectrophotometer. The characteristics of this glass dosimeter for use in soil studies have been described previously (2). In order to determine the absorbed dose and the distribution of absorbed energy within the irradiated soil, the glass dosimeter chips were irradiated through various thicknesses of air-dry soil.

The energy absorption and penetration characteristics in soil are similar to those in water having bulk density of 1 (2).

The urease activity in Dublin soil was examined 5 years after irradiation, but in Hawaii and Puerto Rico soils 1 year after irradiation. The irradiated soils were stored in the original polyethylene bags at room temperature and in air-dry state for the periods indicated.

Results and Discussion

It has been shown before by McLaren et al. (3) that soil urease is resistant to high-energy irradiation and its activity in soil increases upon radiation-sterilization. A series of latosols were subjected to 4 Mrad (twice the nominal sterilization dosage) and to 8 Mrad (4 times the nominal sterilization dosage) 5 Mev electron beam irradiation. The results are shown in Table VIII and in Figure 1.

Sterilizing dosage of 4×10^6 rad (4 Mrad) increased urease activity in both Puerto Rico Nipe clay samples and in the Hawaiian

Table VII

Urease activity in soils compared with soil pH, organic carbon content, and number of microbes.

Soil		urease CO_2 µm/g/hr.	pH	Organic C %	Microorganisms millions/g
Dublin		1.156	5.7	2.75	2.2
Dublin	5 Mrad	2.388			
Kawaihae		0.905	5.9	4.0	4.7
Kawaihae	4 Mrad	1.662			
Kawaihae	8 Mrad	0.695			
Lahaina		0.308	5.7	2.5	0.92
Lahaina	4 Mrad	0.298			
Lahaina	8 Mrad	0.178			
Mahukona		0.083	5.4	2.7	0.34
Mahukona	4 Mrad	0.330			
Mahukona	8 Mrad	0.206			
Molokai		0.040	6.6	1.6	1.0
Molokai	4 Mrad	0.049			
Molokai	8 Mrad	0.010			
Wahiawa		0.154	4.7	2.1	0.081
Wahiawa	4 Mrad	0.033			
Wahiawa	8 Mrad	traces			
Nipe I		0.144	5.1	5.41	0.66
Nipe I	4 Mrad	0.492			
Nipe I	8 Mrad	0.030			
Nipe II		0.113	5.2	5.35	0.27
Nipe II	4 Mrad	0.442			
Nipe II	8 Mrad	0.054			

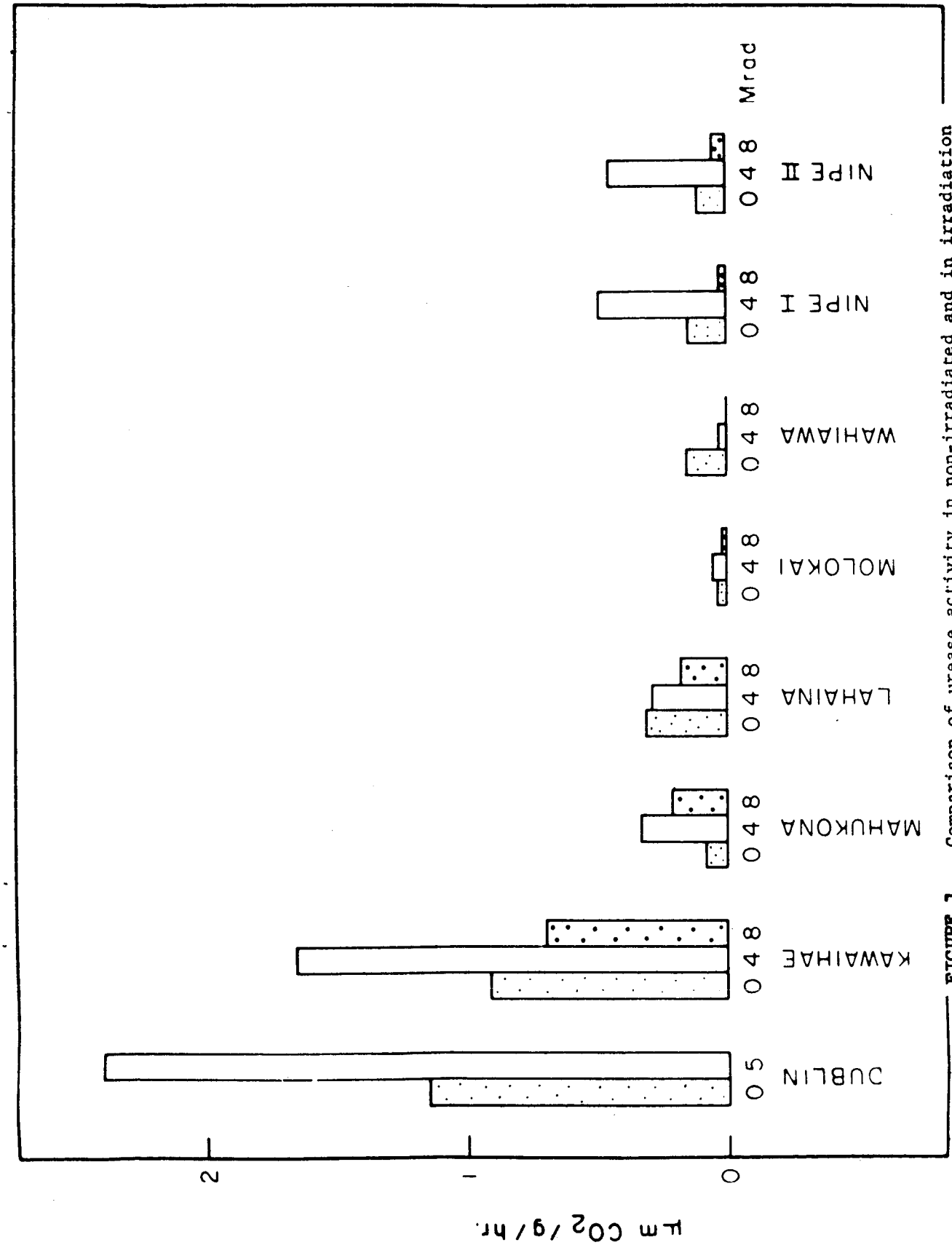

FIGURE 1. Comparison of urease activity in non-irradiated and in irradiation sterilized latosols at 4 Mrad and 8 Mrad doses. Dublin clay irradiated at 5 Mrad dose.

Mahukona, Molokai and Kawaihae soils. Activity decreased slightly in Lahaina, and considerably in Wahiawa soil samples. Doses of 8 Mrad decreased urease activity in all soils below the non-irradiated levels. Dublin soil was examined 5 years after irradiation. It showed a 2.07 times higher activity than non-irradiated duplicates of the same soil collected, stored and otherwise treated the same way.

Evidently, upon irradiation an intracellular soil urease component becomes more accessible to the substrate. The increase may be caused by an unhampered diffusion of substrate and reaction products through the disrupted cell membranes of dead organisms, or due to the free urease released into the soil from the disintegrating organisms.

The relationship of the apparent magnitudes of the soil urease activities at 0, 4, and 8 Mrep doses may be visualized as a sum of several phenomena taking place in the soil during irradiation. In a nonirradiated soil (zero dosage) the apparent urease activity is a sum of extracellular moiety and an intracellular (but, according to the method, not of vigorously proliferating organisms) moiety. Upon irradiation the extracellular moiety is inactivated at a rate $A/A_o = e^{-KD}$, whereas the intracellular moiety becomes available to a finite maximum level to the substrate due to the disruption of cellular membrane integrity, however, being inactivated also at the same rate as the extracellular moiety. Following this scheme it would appear, for example, that most of the urease activity in non-irradiated Nipe clay would be intracellular, whereas in Lahaina clay most of the urease activity would be an extracellular accumulation.

Changes in urease activity in soils during a prolonged air dry storage would follow a similar scheme (1).

We have shown that in a dry urea-urease mixture the enzyme adsorbs water vapor at discrete relative humidities and catalyzes the hydrolysis of urea (1, 4). By subjecting Dublin soil, having its native urease component, to the same experimental conditions, the results showed that hydrolytic activity decreased with decreasing relative humidity but a measurable urea hydrolysis may still be evident at 80% relative humidity (1). As the growth for most of the soil microorganisms ceases around 95% relative humidity (5), the urease activity in soils below this relative humidity level may be atributed to the extracellular enzyme, having the ability to adsorb water-vapor and hydrolyze the substrate similar to that in vitro.

The precise physical and chemical state of extracellular urease in soil is not yet understood, but it is apparent that urease may be adsorbed on surfaces of the colloidal soil particles: in a soil fractionation study the highest urease activity remained associated with a less than 2 μ diameter clay fraction (6). It is also possible that urease may be covalently bound to organic macromolecular soil components. Such hypothesis, of course, needs further verification; however, several authors have reported preparation of catalytically active enzyme derivatives covalently bound to organic polyelectrolyte copolymers, notably by Katchalski and his group (7).

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C. ADSORPTION AND REACTIONS OF CHITINASE AND LYSOZYME ON CHITIN.

The currently reported phase of the investigation of chitinase and lysozyme activity in adsorbed state on chitin is a continuation of the previously described project under the same title (11,16,17,18,19) on enzyme kinetics in structurally restricted systems.

Streptomycete 2B chitinase is adsorbed on chitin in the same amount in the range from pH 4 to pH 10 (16) and the adsorption is not influenced by various ions present over a wide range of concentration. The same amount adsorbs in deionized water as in a 0.2 M NaCl solution (18).

It was observed that dialysis of chitinase solutions against distilled water or weak buffers always resulted in some loss of activity (16). The loss could not be regained by subsequent addition of Na, K, Ca, Mg, Zn or Co salts. The presence of Ca^{++} in low concentrations, however, showed some stimulating effect (18). Repeated experiments confirmed that Ca^{++} has a stabilizing effect on chitinase. Similar Ca^{++} effect has been reported also for other enzymes, e.g., elastase and proteinase (7, 12, 20). It was found also that the purification on DEAE-cellulose at pH 8.9 was more effective than at pH 8.4. Nevertheless, the purified chitinase could be separated further into two fractions on hydroxylapatite column (19).

On the basis of the foregoing, attempts were made to purify the obtained chitinase fractions further in order to obtain enzyme fractions as pure as possible for the use in the study of kinetics of chitinase activity and sorbtive properties.

Materials and Methods

Most of the materials and methods have been described in the previous reports (11,16,17,18,19).

Dispersed chitin. Prepared as described (11).

Chitinase was obtained from the streptomycete strain 2B (11). The modifications in the procedure of preparation are described below in this report.

Absorptivity (extinction coefficient). The value $A_{280}^{mg/ml} = 1.50$ was used for chitinase obtained by the modified procedure; the value $A_{280}^{mg/ml} = 1.10$ was used for chitinase obtained as described before (11,16).

Chitinase activity was based on the amount of the released N-acetylglucosamine, as determined by the DMAB method (11,15).

Proteinase activity was determined by using a modification (6,21) of Kunitz's method (9), as described previously (19).

DEAE cellulose and hydroxylapatite gel (Bio Gel HT) were obtained from Bio-Rad Laboratories, Richmond, California. According to the manufacturer, Bio Gel HT was prepared by the method of Keilin and Hartree (Proc. Roy. Soc. London, Ser. 8, 124, 397 [1938]).

Electrophoresis apparatus - Research Specialities Co., model no. 1400. Total distance along paper strip between the levels of buffer was 48 cm. For each run two 5 x 17 cm oxoid cellulose acetate strips were used with two 11 cm wide Whatman No. 1 filter paper wicks. Distance between wicks: 15.0 cm. Voltages applied: 400 to 600 V. 20 to 200 µg of protein in 0.02 ml distilled water were applied on each strip.

The average duration of each run was 1 hour 40 minutes. Previously used methods (19) were used for herein described work.

Buffers: barbital (veronal), Tris-EDTA-borate, and sodium phosphate have been described (19).

Ponceau S stain, 0.2% in 3% aqueous TCA, used with 200 μg or more protein applied to the electrophoresis strip.

UV light source. A 100 W low pressure Hg lamp, Hanovia SC 37, was used. The light was passed through a 20% acetic acid filter, that retained 184.9 mμ wavelength radiation but did not absorb 253.7 mμ radiation.

Preparation and purification of streptomycete 2B chitinase. The liquid medium and conditions for cultivation of streptomycete 2B have been described (11). It should be noted that slight variations in temperature cause a change in the length of incubation time to reach the maximum chitinase activity in the culture filtrates. Cultures placed on rotary shaker and incubated at 28° - 31° show the highest activity after about 4 days of growth. After the activity peak is reached it drops rather rapidly and as much as 70% of the possible maximum yield of chitinase may be lost by delaying processing for 4 to 5 hours.

The filtrate of streptomycete cultures is clear and at pH 7.4 to pH 7.6.

In order to obtain chitinase with a higher specific activity and a least possible contamination by other proteins, the following purification procedure was developed.

Step I. Concentrated Ca acetate solution was added to culture filtrate to make it 0.0005 M to 0.001 M with respect to Ca^{++}. If $(NH_4)_2SO_4$ precipitation was not followed on the same day, chloroform was added as a preservative, 0.5 ml per liter of filtrate.

Step II. Before $(NH_4)_2SO_4$ precipitation, the pH of filtrate was adjusted to 7.0 with approximately 6 M H_3PO_4. Ammonium sulfate was added to 0.7 saturation. If the precipitate was not collected by centrifugation but on a Buchner funnel over filter paper and Celite pad, the pH of acetate solution should be made up to 8.9 by adding diluted NaOH, as less chitinase was adsorbed on Celite at pH 8.9 than at pH 7.0 (19).

Precipitate was dissolved in cold acetate solution, 0.005 M in Na-acetate and 0.001 M in Ca-acetate, pH 7.0.

Ammonium sulfate from crude chitinase solution was removed by dialysis in the cold. Several drops of 0.01 M $CaCl_2$ or Ca-acetate solution were added to chitinase and dialysed against several changes of acetate solution, pH 7.0, containing 0.005 M Na-acetate and 0.001 M Ca-acetate.

Step III. DEAE-cellulose was washed and prepared as described (11); except, instead of Na-phosphate at pH 8.4, a pH 8.9 solution was used.

Columns were well equilibrated until the pH of the outflowing solution was the same as that of the added Na-phosphate. Actually, at pH 8.9 the phosphate solution has very minimal buffering capacity. The pH of the dialysed crude chitinase solution was brought up to 8.9 before passing it through the column. At this pH chitinase was not adsorbed on DEAE-cellulose but other proteins and colored phenolic compounds were retained as long as pH did not drop below 8.4 and while the exchange capacity of cellulose was not exhausted (19).

After passing through the DEAE-cellulose column chitinase solution became colorless and the pH was adjusted to 7.0 with diluted H_3PO_4, and a drop of 0.01 M $CaCl_2$ or Ca-acetate was added.

Step IV. The final purification of chitinase was done on hydroxylapatite column, equilibrated with 0.001 M Na-phosphate buffer, pH 7.0, and the separation of protein bands was effected by using a concentration gradient of sodium phosphate.

Part of mixed chitinase protein was adsorbed on hydroxylapatite only lightly or not at all. The first activity peak was eluted within limits of 0.001 M and 0.005 M Na-phosphate. The second activity peak of chitinase was eluted with 0.01 M Na-phosphate (19).

Both chitinase fractions from several runs were pooled separately and kept in frozen state for prolonged periods without any change in activity. Similarly, full activity was retained by extensively dialyzed and lyophilized preparations.

Results

The properties of the "first peak" chitinase after purification on hydroxylapatite column were examined and compared with those of the chitinase obtained by ammonium sulfate precipitation and purification on DEAE-cellulose at pH 8.4 and on Sephadex G-50 at pH 7.0.

Absorption spectrum shows a single maximum at 277 mμ (Figure 3). Extinction coeficient was found to be $A_{280}^{mg/ml} = 1.50$.

The chitinase solution was dialyzed cold against several changes of distilled water for about 36 hours at a time. A_{280} of the dialyzed chitinase was 0.482. A 5.0 ml aliquot of dialyzed chitinase solution was dried on a watch glass in a drying oven at 60° for about 7 hours, and kept over P_2O_5 in a desiccator at room temperature for about 2 days. Weight of the dry enzyme protein was 1.600 mg. It gave $A_{280}^{mg/ml} = 1.506$.

Another aliquot of the chitinase solution was similarly dialyzed and lyopilized. The exact weight of a 1 ml weighting glass with lid and of a small glass boat was determined. The lyophilized enzyme was placed in the glass boat and kept in desiccator over night. The pre-weighed boat with protein was placed in the weighing glass, 4.0 ml of glass distilled water was added and the weight determined. Correcting for temperature the exact weight and volume of water was estimated. The dry enzyme protein dissolved in water completely. After determination of absorbancy of 280 mμ the $A_{280}^{mg/ml}$ was computed to be 1.493.

FIGURE 2

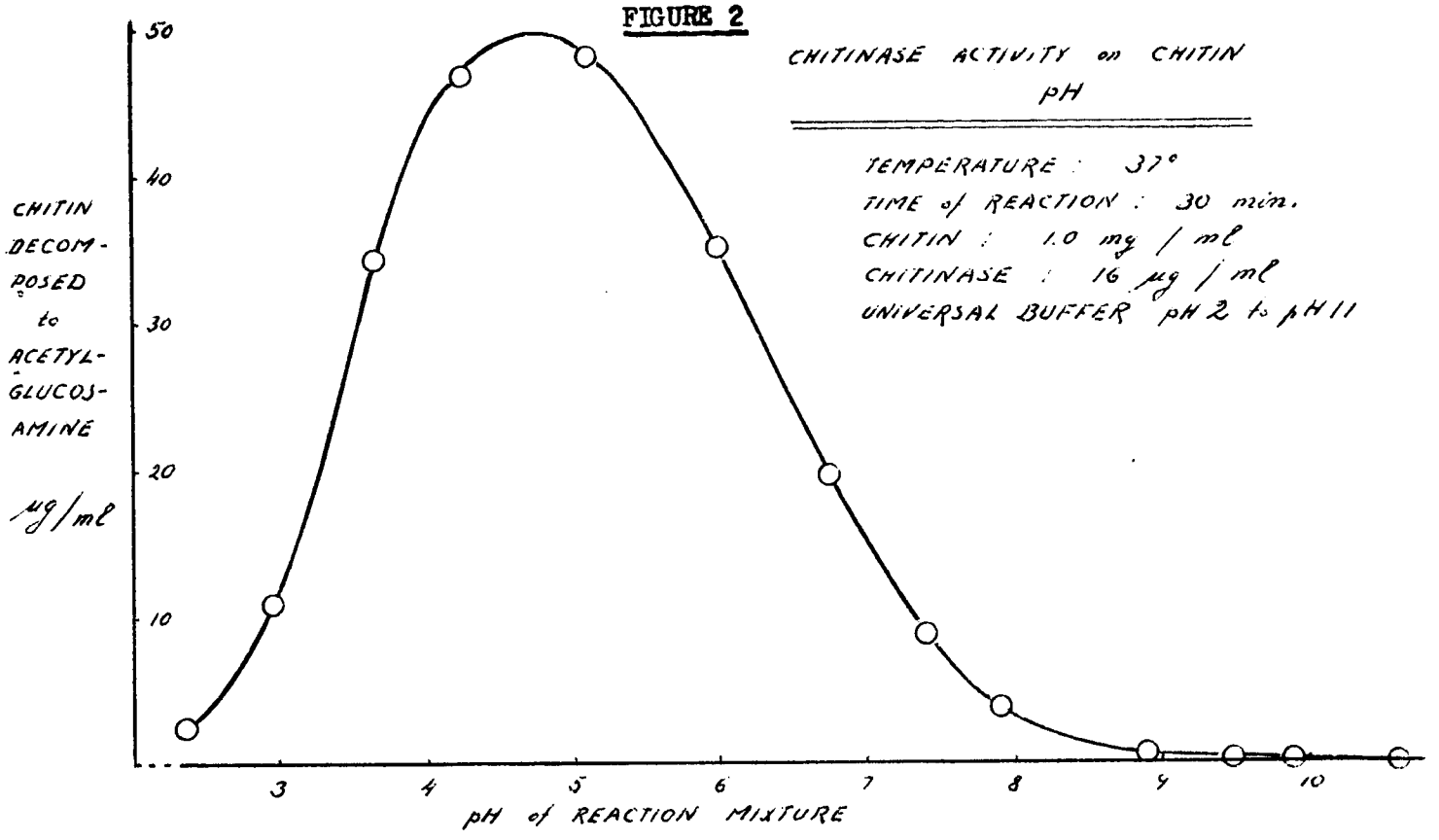
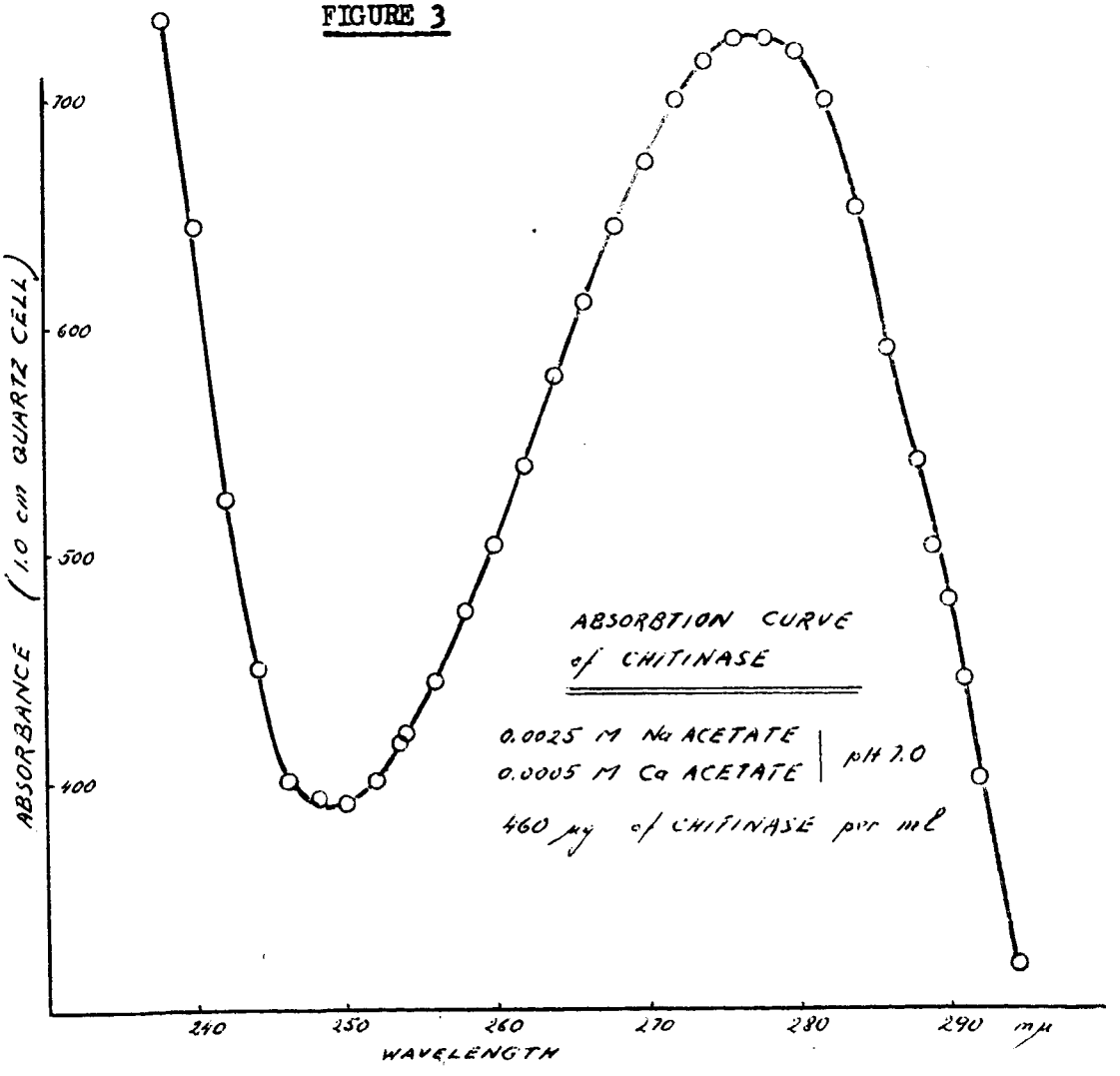


FIGURE 3



The mean value from both measurements was $A_{280}^{mg/ml} = 1.50$. For chitinase obtained by the previously used method, $A_{280}^{mg/ml} = 1.10$. Both absorbancy values were used to estimate the amount of enzyme in corresponding chitinase solutions. The large difference in specific absorbance between the two types of chitinase may be explained by the possible differences in amino acid composition and their relative amounts.

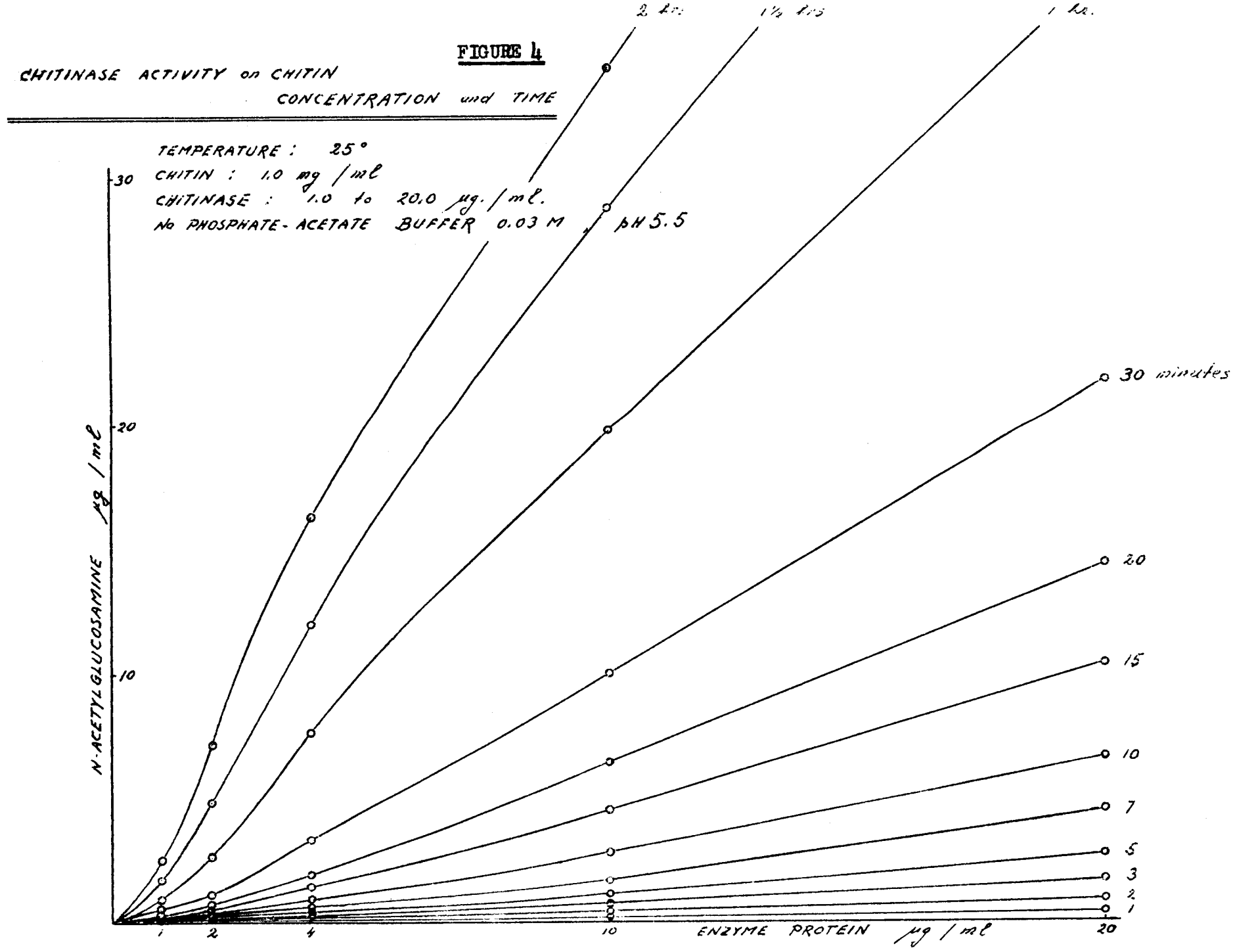
Electrophoretic mobility was tested on cellulose-acetate strips using Tris-EDTA buffer pH 8.6 (1, 19). Only one band was detected, moving toward cathode. In contrast, chitinase prepared by the earlier method separated in 3 bands, all of them moving toward cathode (19).

Activity in the relationship to pH was tested at 25° and at 37°. The highest activity at 37° and in 0.03 M Na-phosphate-acetate buffer was between pH 4.5 and pH 5.0 (Figure 2). At pH 11.0 chitinase was irreversibly inactivated. In comparison with the previously used chitinase the activity pattern vs. pH was the same but the activity value was doubled.

An apparent delay in the hydrolysis rate was apparent, especially at low enzyme concentrations (Figures 4,5,6 and 7). This behavior suggests a possibility that aside from the prevalent random splitting the enzyme preferably would hydrolyze the ends of substrate chains. As the reaction continues there would be more (but shorter) chains available and so more N-acetyl-glucosamine would be released in the same time.

FIGURE 4

CHITINASE ACTIVITY on CHITIN CONCENTRATION and TIME



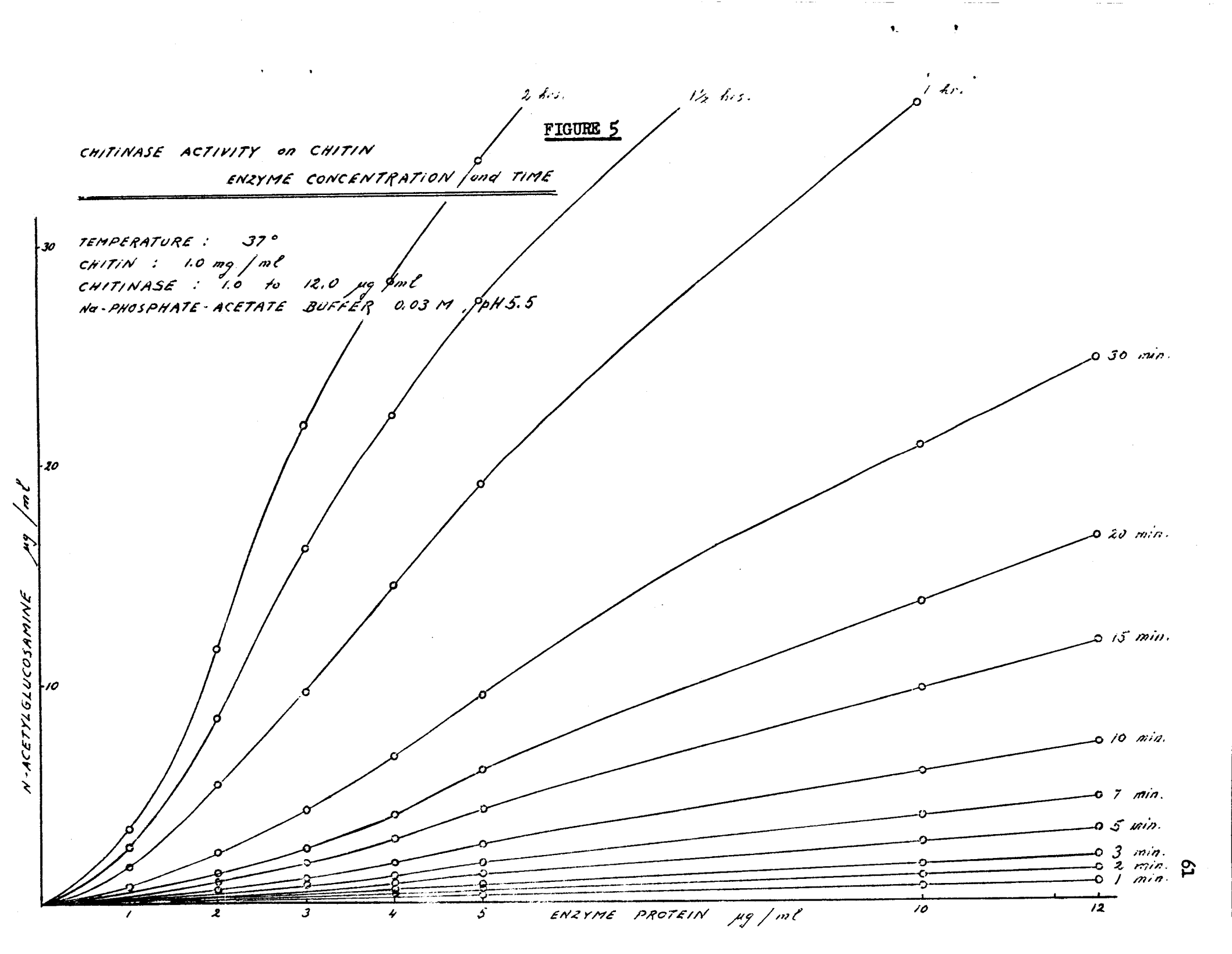


FIGURE 5
CHITINASE ACTIVITY on CHITIN
ENZYME CONCENTRATION and TIME
TEMPERATURE : 37°
CHITIN : 1.0 mg / ml
CHITINASE : 1.0 to 12.0 µg / ml
Na-PHOSPHATE-ACETATE BUFFER 0.03 M , pH 5.5
2 hrs.
1½ hrs.
1 hr.
30 min.
20 min.
15 min.
10 min.
7 min.
5 min.
3 min.
2 min.
1 min.
N-ACETYLGLUCOSAMINE µg / ml
ENZYME PROTEIN µg / ml
30
20
10
1
2
3
4
5
10
12

FIGURE 6

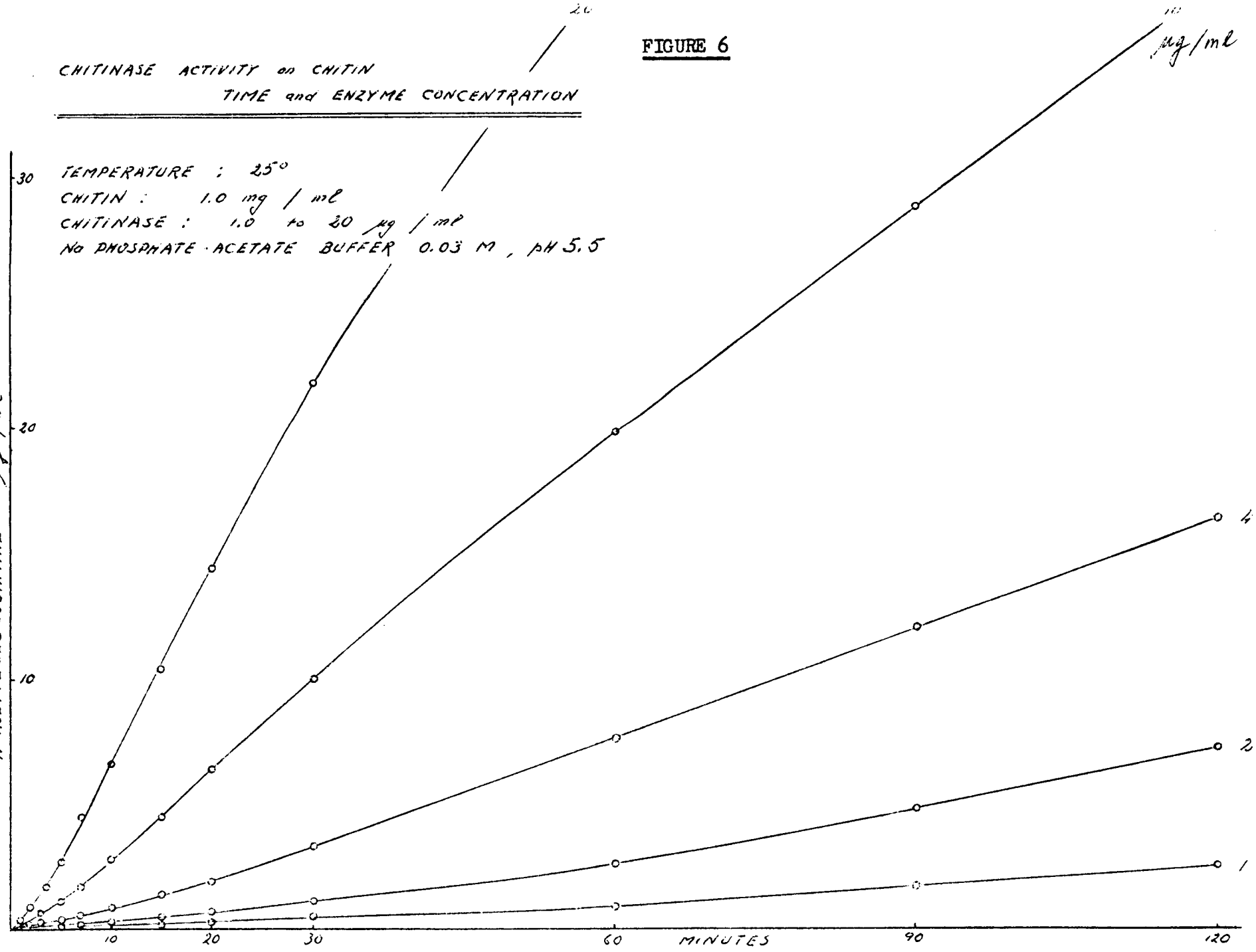
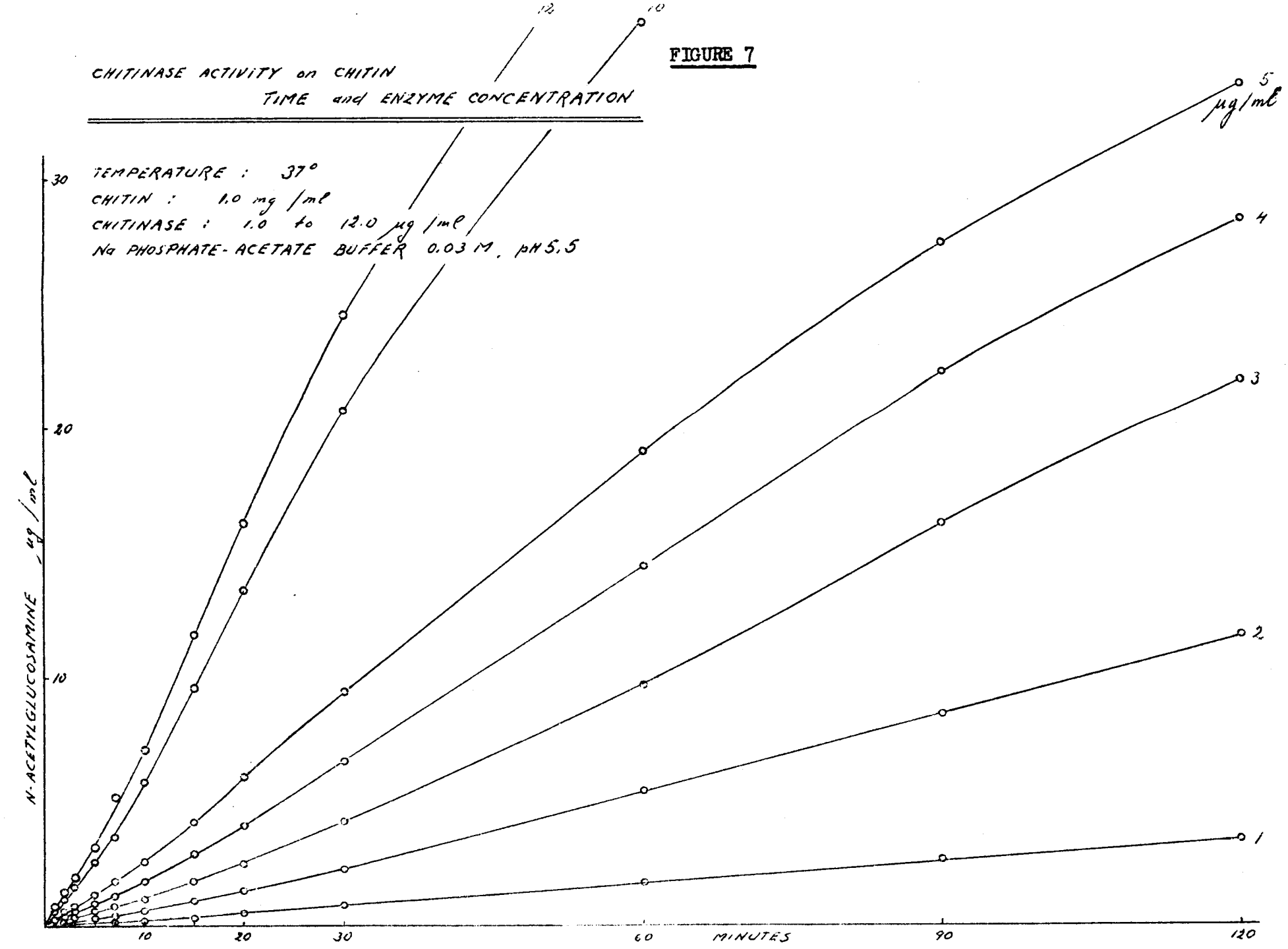

CHITINASE ACTIVITY on CHITIN
TIME and ENZYME CONCENTRATION
TEMPERATURE : 25°
CHITIN : 1.0 mg / ml
CHITINASE : 1.0 to 20 µg / ml
No PHOSPHATE-ACETATE BUFFER 0.03 M, pH 5.5
N-ACETYLGLUCOSAMINE µg / ml
30
20
10
10
20
30
60
90
120
MINUTES
4
2
1
µg/ml

FIGURE 7


CHITINASE ACTIVITY on CHITIN
TIME and ENZYME CONCENTRATION
TEMPERATURE : 37°
CHITIN : 1.0 mg/ml
CHITINASE : 1.0 to 12.0 µg/ml
Na PHOSPHATE-ACETATE BUFFER 0.03 M. pH 5.5
N-ACETYLGLUCOSAMINE µg/ml
30
20
10
10
20
30
60
MINUTES
90
120
12
10
5 µg/ml
4
3
2
1

Both types of chitinase preparations had a considerable proteinase activity (19). Purification on a hydroxylapatite column did not facilitate the separation and proteinase activities.

Stability. The 1.6 mg sample of dry enzyme used for absorbance determination and dried at 60°, was dissolved in 5.0 ml distilled water and centrifuged at high speed. There was no sediment and dissolution of the dried protein was complete. The dissolved sample was tested for activity of 37°. The enzyme (6 µg per ml) released 10.7 µg of N-acetylglucosamine in 30 minutes, compared with 13.0 µg N-acetylglucosamine released by the same amount of non-dialyzed chitinase. The results showed that chitinase had 82% of the original activity retained after dialysis in the cold against distilled water for 36 hours, and subsequent drying in air for 7 hours at 60°. On another occasion after a similar handling 85% of activity was retained. Chitinase, prepared by the previously used method and receiving a similar treatment showed no or only traces of activity (17).

It is likely that not all Ca^{++} can be removed from enzyme protein by dialysis and therefore the enzyme is more stable to inactivation if prepared in presence of Ca^{++}.

Adsorption of chitinase on chitin in a universal buffer medium was the same between pH 4.0 and pH 9.5. No adsorption peak was observed (Figure 8), similarly to the previously prepared chitinase. The amount adsorbed increased in proportion to the enzyme concentration and showed no saturation level (Figure 9). The amount of chitinase adsorbed on chitin in universal buffer was about 18 µg per ml if 250 µg per ml both chitinase and chitin were present. In phosphate-acetate

FIGURE 8

ADSORPTION of CHITINASE on CHITIN
pH

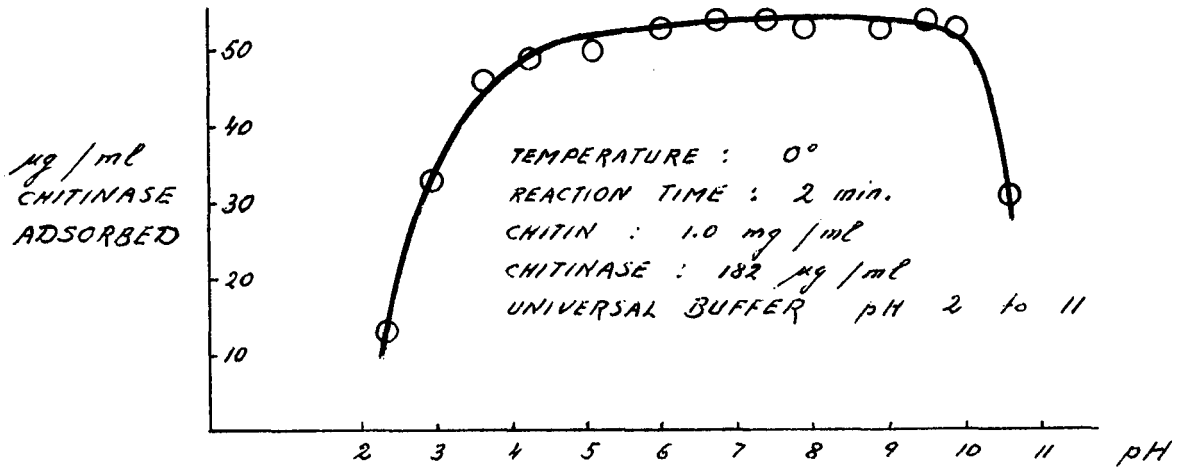
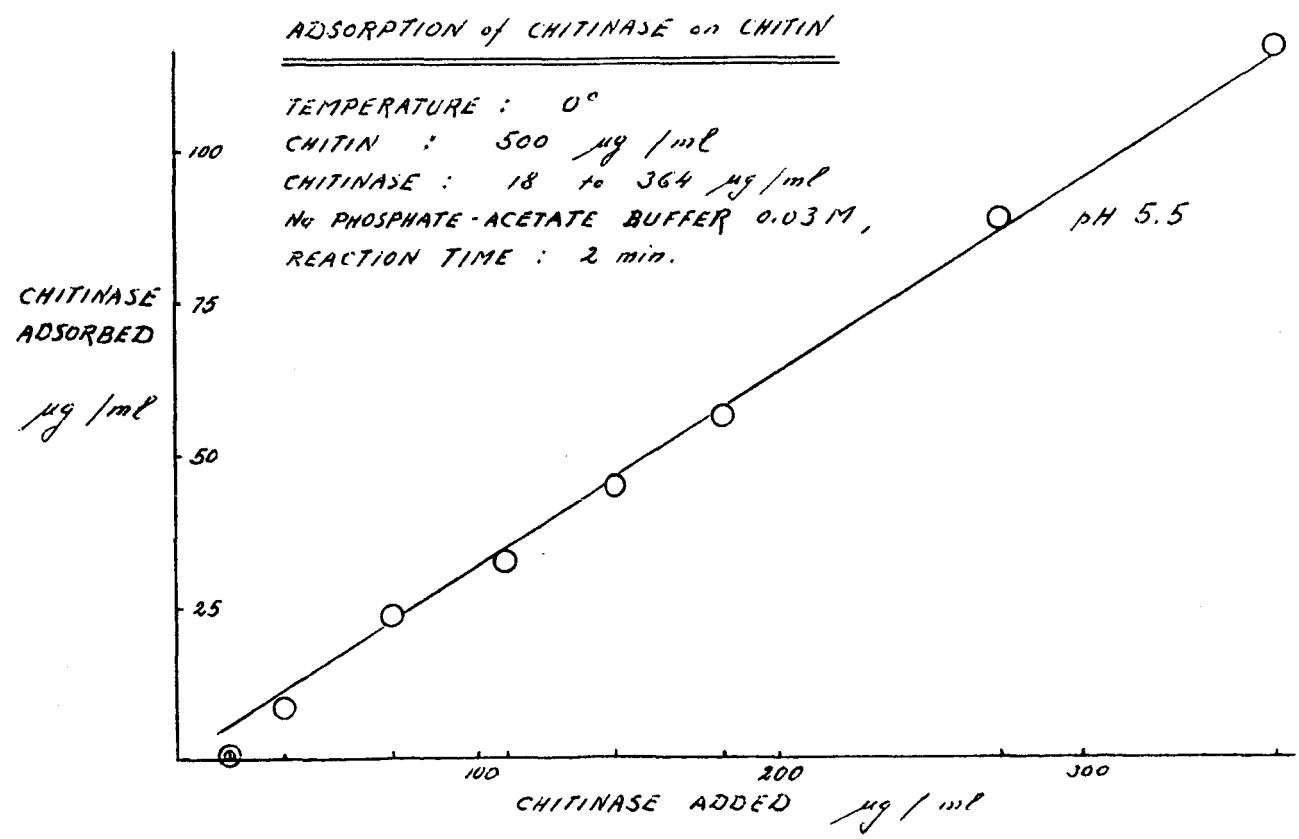


FIGURE 9



buffer the adsorbed amount of chitinase on chitin was 41 μg per ml at the same enzyme and substrate concentrations. At both conditions the previously used chitinase would adsorb about 27 μg per ml (16).

Effect of ultraviolet irradiation. In all adsorption experiments the amount of chitin is rapidly decreasing due to hydrolysis by chitinase. For each measurement the released N-acetylglucosamine was determined and the actual amount of chitin present during the experiment was computed. Furthermore, certain changes in the sorptive characteristics of chitin while undergoing degradation may take place. It would be of value to compare the observed adsorption values with those of an inactivated chitinase but having an unimpaired ability to adsorb on chitin. We have demonstrated that heat inactivated chitinase did not adsorb on chitin (17). Preliminary experiments were performed to demonstrate whether an inactivated but adsorbing chitinase could be obtained by using ultraviolet irradiation.

Chitinase solution (910 μg per ml) was pipetted into a parallel-sided round quartz cell and was kept under constant stirring while being irradiated. Some inactivation was noticed after 20 minutes of UV irradiation; after $2\frac{1}{2}$ hours of exposure 16% of activity was lost. Adsorption of $2\frac{1}{2}$ hours irradiated chitinase on chitin was 96% as compared with controls.

Apparently a more intense irradiation source and procedure is needed for a complete radiation-inactivation of chitinase.

Discussion

There is no doubt that chitinase system of streptomyces is a complex one (2-5,8,10,11,14,15-19,22). It is not clear, however, whether the differences in properties of chitinase from different streptomycete strains are due to various relative amounts of a number of specific chitinases in respective organisms, or, whether there are differences in protein structures, and, consequently, in enzyme specificities. Often the enzyme proteins of various species chemically are closely related, and distinguishable only by immunological methods (23).

The chemical and physical characteristics, behaviour and homogeneity of any chitinase preparation is wholly dependent on the "art" of isolation and methods for the determination of activity (i.e., activity measurements based on DMAB color reaction or on decrease in turbidity may give widely differing results). There have been no reports in literature regarding crystallization of chitinase as yet.

The following complicating factors, among others, were evident in our work:

1. Ammonium sulfate fractionation yielded several fractions of chitinase with different properties, obtained by varying the pH and the ammonium sulphate concentration.
2. It is only generally true that chitinase is not adsorbed on DEAE-cellulose between pH 8.4 and pH 8.9. Also, during fractionation several activity peaks having chitinase and chitobiase activities may be recovered.

At pH 8.4 to pH 8.2 on DEAE-cellulose column another enzyme

protein fraction is released that shows chitinase activity and may be separated later on sephadex G-50 column. This enzyme protein may be lost completely if the pH in the DEAE-cellulose column is not permitted to drop slightly below pH 8.4 (19).

3. A complete separation of chitinase and proteinase activties have been achieved only in few cases (8). The method described herein does not remove proteinase activity from chitinase, and proteinase activity is present also in the "second activity peak" chitinase from hydroxylapatite column (19).

Nevertheless a considerable amount of information has been published in the literature regarding chitinase (*cf.* listing, ref. 19) to facilitate a thorough and detailed examination of chitinase systems.

Summary

A modified procedure for preparation and purification of the streptomycete chitinase system is described where Ca^{++} is used as a stabilizing agent. A homogenous enzyme preparation was obtained. Several properties of the obtained chitinase were compared with those of a more heterogeneous chitinase preparation used previously.

It was found that $A_{280}^{mg/ml} = 1.50$. Electrophoresis in Tris-EDTA buffer, pH 8.6, showed only one band moving toward cathode. The highest activity was at pH 4.5 to 5.0 and the activity was irreversibly destroyed

at pH 11.0. Adsorption on chitin was dependent on the enzyme concentration but not on the pH (between pH 4.0 and 9.5). With 250 µg of each enzyme and substrate present in a 0.03 M Na-phosphate-acetate buffer at 0°, about 40 µg of chitinase protein was adsorbed on chitin. The higher activity and stability was attributed to the presence of Ca^{++} during purification procedures. The enzyme showed also a high resistance against UV irradiation.

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